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Effect of 4-Dimethylaminomethyl-1-(3-hydroxyphenyl)-1-nonen-3-one Hydrochloride and Related Compounds on Respiration in Rat Liver Mitochondria

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Abstract □ 4 - Dimethylaminomethyl - 1 - (3 - hydroxyphenyl) - 1- nonen 3-one hydrochloride (II) was shown to stimulate respiration in rat liver mitochondria at levels of 2.5 μmoles or less; but at levels higher than 5.0 μmoles, respiration was inhibited when succinate and 3-hydroxybutyrate were the substrates. Compound II caused inhibition of respiration in the presence of glutamate over the dose range studied. The stimulating effect of II was attributed to its functioning as an uncoupling agent. Its inhibiting properties were considered to be due to its behaving like antimycin A in blocking transport of electrons between cytochromes b and c_1. The effect of II on respiration in mitochondria varied with the pH of the medium. A conjugated styryl ketone, which contained a nuclear hydroxy function and was structurally related to II, also stimulated respiration at low doses while inhibiting respiration at higher concentrations. Etherification of the hydroxy group led to compounds in which only stimulation of respiration was noted.

Keyphrases □ Respiration, mitochondria—effect of 4-dimethylaminomethyl-1-(3-hydroxyphenyl)-1-nonen-3-one hydrochloride and styryl ketone derivatives □ Mannich bases—prepared from styryl ketones, effect on respiration in mitochondria □ Styryl ketones—preparation of Mannich bases

A number of Mannich bases (I) derived from styryl ketones containing chlorine or hydrogen atoms in the aromatic ring have been shown to inhibit the electron transport chain in mitochondria of both rat liver cells and yeast (1, 2). These compounds were considered to exert their effect by competition with coenzyme Q_{10} (1). Recently, 4-dimethylaminomethyl-1-(3-hydroxyphenyl)-1-nonen-3-one hydrochloride (II), which is a Mannich base with a nuclear hydroxy group, was synthesized (3). Although it had little action against murine P-388 lymphocytic leukemia in contrast to some dichlorinated derivatives of I (4), it displayed anti-inflammatory analgesic properties as well as antifungal activity (3).

Therefore, the questions were asked whether II would affect mitochondrial function and, if so, whether its site of action would differ from that in series I. In addition, if II affected the electron transport chain, which is found in the inner membrane of the mitochondria, it could be a carrier group to which other molecular entities may be attached *via* the hydroxyl group. Furthermore, two related α,β-unsaturated ketones, III*a* and III*d*, differed in their activity towards P-388 lymphocytic leukemia (3), which could be due to a variation in effect on mitochondria. Thus, it was of interest to examine the effects of II, III*a*, III*d*, and the related compounds III*b* and III*c* on the electron transport chain in rat liver mitochondria.

DISCUSSION

Table I indicates the effect of II on respiration in rat liver mitochondria using three different substrates. In the case of succinate and 3-hydroxybutyrate, respiration was stimulated at low concentrations; at higher doses of II, inhibition of respiration was noted. When glutamate was the substrate, no stimulation of respiration was noted and only inhibition of respiration was found over the dose range studied.

The inhibiting effect of II at 5 μmoles with succinate as the substrate was considered. The related compounds I*a* and I*b* caused inhibition of respiration by competition with coenzyme Q_{10}, which could be reversed by addition of coenzyme Q_{10} (1). Therefore, a possible site for the respiration-inhibiting action of II might be similar. However, addition of coenzyme Q_{10} to mitochondria whose respiration had been inhibited by II did not alter the percentage of respiration inhibition, so the mode of action of II appears to differ from that of I.

A number of compounds inhibit respiration by blocking the transport

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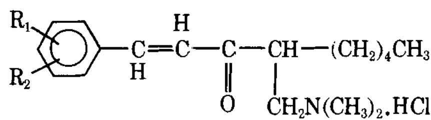
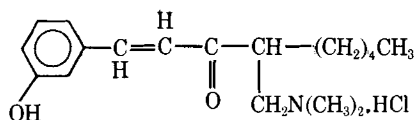
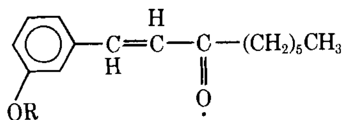


I: $R^1 = R^2 =$ H, Cl



II



IIIa: R = $CH_2OC_2H_5$
IIIb: R = CH_2OCH_3
IIIc: R = CH_3
IIId: R = H

of electrons along the cytochromes. On occasion, this blockade and cytochromes may be discerned by examining the difference in the visible spectra of cytochromes in both the oxidized and reduced states (5). During respiration, electrons are transported along the cytochromes from coenzyme Q_{10} to oxygen (Scheme I), and the cytochromes become reduced during this process. After a blockade has occurred, the cytochromes prior to the block become reduced and the cytochromes after the block become more oxidized due to the pseudoreversibility of the respiration chain.

Examination of the visible spectra of II showed the presence of cytochrome b with its Soret band at 430 nm and its α band at 563 nm, suggesting a block between cytochromes b and c_1. The visible spectra of antimycin A was identical to that obtained for II. Since antimycin A is known to block between cytochromes b and c_1 (6), a similar mode of action for II was suggested. To confirm the site of action, respiration was examined in the presence of an inhibiting concentration of II, and then tetramethyl-*p*-phenylenediamine was added, which caused respiration to return to 95% of the rate prior to the addition of II. This result suggests that the block by II occurs between cytochromes b and c_1. Antimycin A was shown to act in a similar manner. After respiration was inhibited by this compound, tetramethyl-*p*-phenylenediamine caused the respiration rate to return to normal.

The reason for stimulation of respiration by II at low doses was also investigated with succinate as the substrate. Since II and coenzyme Q_{10} are α,β-unsaturated ketones, both compounds possibly might act as recipients of electrons from various flavoproteins (Scheme I), and these electrons are subsequently transferred to the cytochromes. Coenzyme Q_{10} was removed from the mitochondria; in the presence of succinate, neither succinoxidase nor succinate cytochrome c reductase was active, but activity was partially restored by the addition of coenzyme Q_{10}. Substitution of II for coenzyme Q_{10} did not permit respiration to occur, and it seems unlikely, therefore, that II acts in a capacity similar to this coenzyme. This finding substantiates the observation of earlier workers that a number of electron acceptors related to coenzyme Q_{10} failed to stimulate its action (7) and that mitochondria may have a specific requirement for this coenzyme.

Table I—Effect of II on Respiration in Rat Liver Mitochondria Using Succinate, Glutamate, and 3-Hydroxybutyrate as the Substrates[a]

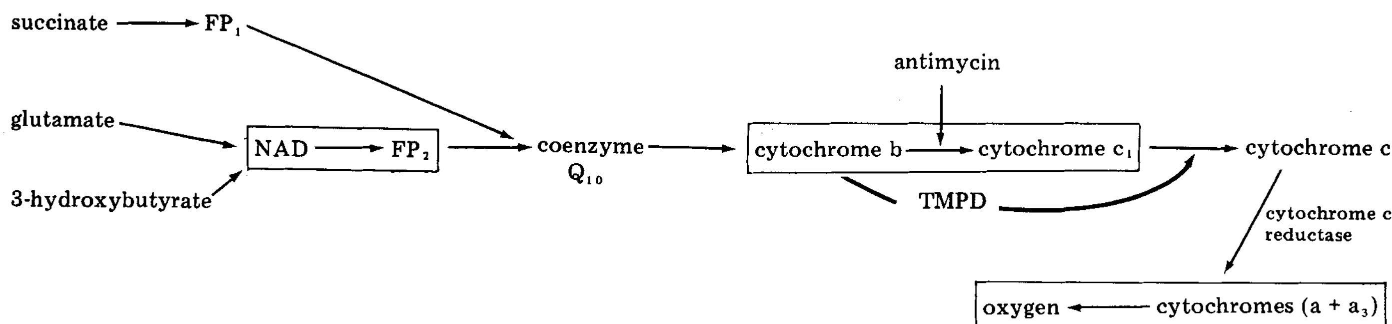
	Substrate		
Concentration, µmoles	Succinate[b] Inhibition, % ± *SE*	Glutamate[c] Inhibition, % ± *SE*	3-Hydroxybutyrate[d] Inhibition, % ± *SE*
0.05	−18.80 ± 3.18	1.70 ± 1.70	−15.45 ± 3.20
0.1	−28.64 ± 7.52	0.00 ± 0.00	−21.16 ± 1.85
0.25	−47.90 ± 0.62	6.65 ± 1.60	−48.03 ± 4.01
0.5	−59.53 ± 11.38	17.80 ± 4.22	−48.10 ± 2.64
1.0	−75.34 ± 11.85	49.26 ± 6.24	−57.88 ± 10.77
1.25	−77.68 ± 6.17	51.28 ± 9.87	−41.93 ± 1.65
2.5	−36.06 ± 3.20	62.08 ± 0.95	83.10 ± 1.42
5	73.43 ± 1.72	66.17 ± 2.28	98.04 ± 1.11
10	97.43 ± 0.72	81.52 ± 2.51	—

[a] Figures prefaced by a negative sign indicate stimulation of respiration. [b] At a concentration of 0.125 µmole, stimulation of respiration was 29.78% (*SE* = 8.09). At concentrations of 3.75, 4.25, 7.5, and 12.5 µmoles, the percentage inhibition of respiration was 8.34 (*SE* = 3.74), 49.52 (*SE* = 5.30), 58.87 (*SE* = 1.65), 92.79 (*SE* = 1.92), and 99.70 (*SE* = 0.30), respectively. [c] At concentrations of 0.75, 15, 20 and 25 µmoles, the percentage inhibition was 13.70 (*SE* = 6.35), 81.13 (*SE* = 3.61), 85.50 (*SE* = 1.80), and 86.87 (*SE* = 1.13), respectively. [d] At a concentration of 0.75 µmole, stimulation of respiration was 58.88% (*SE* = 5.50).

Increased respiration in mitochondria could occur by II acting as an uncoupling agent, especially since II is a phenolic compound and some substituted phenols (such as 2,4-dinitrophenol) are known to be uncoupling agents. In the case of 2,4-dinitrophenol, respiration is stimulated at low concentrations and inhibited at higher concentrations (8, 9), which is a situation similar to that found with II. Uncouplers do not interfere with the transport of electrons from the flavoproteins to oxygen, but adenosine triphosphate (ATP) is not formed during this process, *i.e.*, oxidation and phosphorylation no longer function in unison. Uncouplers may be identified by their effect on the P:O ratio, that is, the number of moles of adenosine diphosphate (ADP) phosphorylated to produce adenosine triphosphate per gram atoms of oxygen consumed (10). In tightly coupled mitochrondria with succinate as the substrate, such a ratio should be 2 (Scheme I).

The effect of II on the P:O ratio was examined, and it was found that utilization of inorganic phosphate decreased while oxygen consumption was unchanged from that of the control. This result caused the P:O ratio to fall to 25% of the value obtained in the absence of II. As well as uncoupling of oxidative phosphorylation (11), 2,4-dinitrophenol and some related phenols also stimulate latent adenosine triphosphatase activity of normal mitochondria (12, 13). If II acted in the same manner as 2,4-dinitrophenol, one would expect to see stimulation of adenosine triphosphatase along with the uncoupling effects, *i.e.*, a decreased P:O ratio. It was found that low concentrations of II (0.20–2.00 µmoles) caused stimulation of adenosine triphosphatase activity and a subsequent increase in hydrolysis of adenosine triphosphate to adenosine diphosphate. Higher concentrations of II (3 µmoles or greater) caused inhibition of this enzyme.

In a study of a series of uncoupling agents, 2,4-dinitrophenol caused stimulation and subsequent inhibition of respiration in the presence of both succinate and glutamate. However, in the case of the uncoupler 3-nitrophenol, stimulation of mitochondrial respiration occurred in the presence of succinate but not glutamate, a situation identical to that found with II (8). With both 3-nitrophenol and II, electron-attracting groups are present in the *meta* position to the phenolic function, which may reflect similar structural requirements for the mode of action of these two compounds.



Scheme I—The rectangles indicate the sites where ADP is converted into ATP. Flavoproteins are represented as FP_1 and FP_2.

Table II—Effect of II on Respiration in Rat Liver Mitochondria Using Succinate as the Substrate at pH Levels of 6.4, 6.9, and 7.4 [a]

Concentration, µmoles	pH 6.4[b] Inhibition, % ± *SE*	pH 6.9[c] Inhibition, % ± *SE*	pH 7.4[d] Inhibition, % ± *SE*	Levels of Significance (p) between pH Values of 6.4 and 6.9	6.9 and 7.4	6.4 and 7.4
0.25	−32.07 ± 10.79	−42.51 ± 2.74	−47.90 ± 0.62	0.50	0.05	0.10
0.50	−43.06 ± 1.13	−55.67 ± 0.01	−59.53 ± 11.38	0.02	>0.50	0.50
1.00	−33.31 ± 1.07	−94.06 ± 1.51	−75.34 ± 11.85	0.001	0.50	0.10
1.25	−72.72 ± 8.97	−61.64 ± 12.79	−77.68 ± 6.17	0.50	0.50	>0.05
2.50	−56.33 ± 13.10	−55.14 ± 3.91	−36.06 ± 3.20	>0.50	0.10	0.10
4.25	+4.81 ± 9.86	+81.63 ± 7.26	+49.52 ± 5.30	0.01	0.05	0.01
4.50	+11.97 ± 3.25	+84.45 ± 4.34	+58.87 ± 1.65	0.01	<0.001	<0.001
5.00	+52.85 ± 6.35	+92.55 ± 1.63	+73.43 ± 1.72	0.01	<0.001	0.01
7.50	+98.25 ± 1.76	+97.62 ± 2.38	+92.79 ± 1.92	>0.50	0.50	0.10
10.00	+99.08 ± 0.93	+99.38 ± 0.62	+97.43 ± 0.72	>0.50	0.50	0.50

[a] Figures prefaced by a negative sign indicate stimulation of respiration. [b] At a concentration of 0.75 µmole, stimulation of 28.13% (*SE* = 0.85) occurred. At a concentration of 6.00 µmoles, inhibition of 65.80% (*SE* = 6.92) occurred. [c] At a concentration of 0.75 µmole, stimulation of 60.22% (*SE* = 0.95) occurred. At a concentration of 3.50 µmoles, inhibition of 37.27% (*SE* = 12.71) occurred. [d] At concentrations of 0.05, 0.10, and 0.125 µmoles, stimulation of respiration was 18.80% (*SE* = 3.18), 28.64% (*SE* = 7.52), and 29.78% (*SE* = 8.09), respectively. At concentrations of 3.75 and 12.50 µmoles, the percentage inhibition of respiration was 8.34 (*SE* = 3.74) and 99.70 (*SE* = 0.30), respectively.

A recent study revealed that the amount of I inhibiting respiration in mitochondria obtained from both normal rat liver and from a Morris hepatoma varied according to the pH of the medium (14). In general, the compounds had increased respiration-inhibiting properties as the pH was lowered. The three pH values chosen were 7.4, that of normal physiological conditions; 6.9, which is claimed to be the pH of certain tumors (15, 16); and 6.4, which has been reported to be the pH of some tumors after administration of glucose while normal tissue was unaffected (15, 16). Thus, the question was raised as to whether the effect of II would vary markedly with such pH changes. If respiration was inhibited, preferentially at lower pH levels, some selectivity towards tumors may be possible under certain conditions.

Table II shows that at the lower stimulating concentrations of II, the percentage increase in respiration generally fell as the pH of the medium was lowered. This respiration-stimulatory effect became less pronounced as inhibiting concentrations of II were approached. When inhibition of respiration occurred at concentrations greater than 2.50 µmoles, there was mainly an optimal inhibition of respiration at pH 6.9 but less inhibition at pH 6.4 than at pH 7.4. Hence, these results of inhibition differ from those reported for series I (14).

Finally, the conjugated styryl ketones (III), which are related to II, were evaluated for their effect on respiration in rat liver mitochondria (Table III). At the optimal dose levels, II and III*a*–III*d* increased the survival time in mice with P-388 lymphocytic leukemia by 6, 25, 13, 5, and 4%, respectively; little correlation between effect on respiration and anticancer activity was seen. The ethers III*a*–III*c* caused moderate stimulation of respiration over the dose range studied, and no inhibition of respiration was noted. However, at concentrations of >1.0 µmole, for example, inhibition of respiration may occur at the same time as stimulation but the latter effect predominates. The phenolic compound III*d* behaves in a similar manner to the corresponding Mannich base II, and hence the observed inhibition of respiration appears to be associated with the presence of a phenolic group.

This study has shown that a Mannich base derived from a nuclear hydroxy conjugated styryl ketone (II) has uncoupling action at low concentrations while at higher doses it acts like antimycin A in blocking electron transport between cytochromes b and c_1. The effect on mitochondria varies depending on the pH of the medium. While III*d* showed a similar biphasic response as II, etherification of the hydroxy group of III*d* produced derivatives III*a*–III*c*, which showed only slight stimulating effects on respiration.

EXPERIMENTAL[1]

Syntheses of Compounds—Compounds II, III*a*, and III*b* were prepared according to literature methodology (3). (*E*)-1-(*m*-Methoxymethyloxyphenyl)-1-nonen-3-one (III*b*), prepared by the same scheme as III*a* in a 58% yield, was a yellow oil, bp 187°/0.3 mm [lit. (17) bp 177°/0.16 mm].

Anal.—Calc. for $C_{17}H_{24}O_3$: C, 73.88; H, 8.75. Found: C, 73.42; H, 8.70.

(*E*)-1-(*m*-Methoxyphenyl)-1-nonen-3-one (III*c*) was prepared as follows. A mixture of 2-octanone (5.13 g, 0.04 mole), *m*-methoxybenzaldehyde (4.42 g, 0.0325 mole), and sodium hydroxide (0.68 g, 0.017 mole) in water (20 ml) was heated under reflux for 24 hr with mechanical stirring. On cooling, the aqueous layer was extracted three times with benzene (20 ml), and the combined organic phases were washed twice with water (20 ml) and dried with anhydrous magnesium sulfate. Removal of benzene by a water aspirator afforded a yellow oil, which was purified by distillation to give III*c* as a colorless oil, bp 144–146°/0.35 mm, in a 52% yield.

Anal.—Calc. for $C_{16}H_{22}O_2$: C, 78.01; H, 9.00. Found: C, 77.94; H, 8.98.

Effect of II and III*a*–III*d* on Mitochondria Isolated from Rat Livers—Pellets of mitochondria obtained from the livers of male Wistar rats were isolated by the literature methodology (1) and suspended in 3.4 m*M* tromethamine hydrochloride[2] buffer (pH 7.40) containing 0.25 m*M* sucrose and 1 m*M* ethylene glycol bis(aminomethyl)tetraacetate at 0°. The protein content was 4 mg/ml as determined by a modification (18) of a previously reported procedure (19). The medium for the determination of mitochondrial respiration was that described previously (1) and will be referred to subsequently as the respiration media. Compounds III*c* and III*d* were dissolved in absolute ethanol and II in 10 m*M* tromethamine hydrochloride buffer (pH 7.40), while the oils III*a* and III*b* were added directly to the mitochondrial suspension. The media and methodology required for generating the data recorded in Tables I and II were those employed previously (1) using an oxygen monitor[3]. At least four determinations per compound were obtained at each dose level.

Effect of II and Antimycin A on Visible Spectra of Cytochromes—A mitochondrial suspension (0.5 ml containing 4 mg protein/ml) was placed in two 1-cm cells containing the respiration media (pH 7.40, 2.5 ml). Solutions of disodium succinate (15 µmoles) in 7.5 µl of 10 m*M* tromethamine hydrochloride buffer (pH 7.4) and adenosine diphosphate (1 µmole) in 1 µl of 10 m*M* tromethamine hydrochloride were placed in each cell. One cell was aerated by agitation while toluene was added to the other one. The visible spectra[4] of oxidized and reduced cytochromes were then recorded between 390 and 590 nm.

The two cells containing mitochondria, disodium succinate, and adenosine diphosphate in the respiration media (pH 7.4) were aerated by agitation. Compound II (5.0 µmoles in 10 m*M* tromethamine hydrochloride buffer, pH 7.4) was added to one cell and allowed to stand at room temperature for 3 min. Just before the spectrum was recorded, II was added to the reference cell. This addition eliminated the interference caused by II with the difference spectrum. The spectrum was then recorded between 590 and 490 nm. A fresh reference cell was prepared and aerated, II was added, and the spectrum recorded between 490 and 390 nm. All determinations were carried out at room temperature.

Mitochondria, disodium succinate, and adenosine diphosphate in the respiration media were added to the cells in the quantities indicated above. Antimycin A[2] (0.009 µmoles in 2 µl of 10 m*M* tromethamine hydrochloride buffer, pH 7.4) was added to one cell; after both were aerated, the difference spectra were recorded.

Effect of II and Antimycin A on Tetramethylphenylenediamine Bypass—Mitochondria (0.5 ml containing 4 mg protein/ml) were added to the respiration media (2.5 ml) in the cell of an oxygen monitor at 37°, and uptake of oxygen started with the addition of disodium succinate

[1] Elemental analyses were carried out by Mr. R. E. Teed, Department of Chemistry and Chemical Engineering, University of Saskatchewan. Boiling points are uncorrected.

[2] Sigma Chemical Co., St. Louis, MO 63178.
[3] Model 53, Yellow Springs Instrument Co., Yellow Springs, OH 45387.
[4] Cary 15M spectrometer, Varian Instrument Division, Monrovia, CA 91016.

Table III—Effect of IIIa–IIId on Respiration in Rat Liver Mitochondria Using Succinate as the Substrate [a]

Concentration, μmoles	IIIa Stimulation, % ± SE	IIIb [b] Stimulation, % ± SE	IIIc Stimulation, % ± SE	IIId [c] Stimulation, % ± SE
0.01	7.93 ± 6.32	7.23 ± 2.87	12.09 ± 4.77	26.43 ± 6.46
0.1	25.75 ± 1.30	14.99 ± 5.10	20.74 ± 4.26	86.07 ± 13.64
1.0	27.98 ± 8.35	22.74 ± 8.89	25.45 ± 16.09	−47.23 ± 7.31
2.5	27.38 ± 10.70	13.45 ± 5.29	5.59 ± 2.52	−87.56 ± 4.63
5.0	20.18 ± 6.00	8.19 ± 3.30	14.63 ± 5.92	—
50.0	5.72 ± 3.66	16.25 ± 1.95	23.74 ± 5.37	—

[a] Figures prefaced by a negative sign indicate inhibition of respiration. [b] At a concentration of 10 μmoles, stimulation of 11.39% (*SE* = 1.13) occurred. [c] At a concentration of 5.0 μmoles or greater, 100% inhibition occurred.

(15 μmoles in 7.5 μl of 10 m*M* tromethamine hydrochloride buffer, pH 7.4). Respiration was inhibited by either antimycin A (0.009 μmole) or II (5.0 μmole), and then tetramethyl-*p*-phenylenediamine (0.013 μmole) in 10 m*M* tromethamine hydrochloride buffer, pH 7.4 (25 μl), was added. The change in the rate of oxygen uptake was recorded.

Effect of II on Succinoxidase and Succinate–Cytochrome c Reductase Reactions—Acetone-treated particles were prepared from rat liver mitochondria by the literature method (20), and 0.5 ml of a suspension of these particles (3 mg protein/ml) was placed in the cell of an oxygen monitor containing monobasic potassium phosphate (100 μmoles), disodium succinate (150 μmoles), cytochrome c[5] (0.2 mg), and sufficient sucrose solution to produce 3 ml of a 0.2 *M* solution with a pH of 7.4. After 9-min incubation at 37°, various quantities of coenzyme Q_{10}[2] solutions (4 mg in 2 ml of ethanol, 95% w/v) and II (1.25 μmoles in 2.5 μl of 10 m*M* tromethamine hydrochloride buffer, pH 7.4) were added, and the effect on oxygen uptake was recorded. This experiment was carried out four times.

A mixture of the acetone-treated particles (10 μl, 3 mg protein/ml), monobasic potassium phosphate (180 μmoles), and cytochrome c (15 mg), with or without II and coenzyme Q_{10} (20 μg) in a total volume of 3 ml of water, was placed in the cell of an oxygen monitor. After incubation at 30° for 10 min, 0.1 ml of a solution containing disodium succinate (135 μmoles) and potassium cyanide (3 μmoles) in water was added, and the reaction was monitored at 550 nm[6] for 30 min. This determination was carried out in duplicate.

Effect of II on P:O Ratio—The P:O ratio of aerobic phosphorylation of mitochondria in the presence of II (1.25 μmole) was carried out at 30° using the literature methodology (21). The quantity of inorganic phosphate was determined by the method described previously (22). In an experiment involving three separate determinations, 1.43 (±0.38 *SE*) μmoles of inorganic phosphate were taken up, and 1.05 (±0.03 *SE*) μatoms of oxygen was consumed in the absence of II, indicating a P:O ratio of 1.36. In the presence of II, 0.26 (±0.14 *SE*) μmole of inorganic phosphate and 1.03 (±0.11 *SE*) μatoms of oxygen were utilized, indicating a P:O ratio of 0.25, *i.e.*, 18% of the P:O ratio of the control value.

In a second experiment involving three separate determinations, 1.33 (±0.30 *SE*) μmoles of inorganic phosphate and 0.685 (±0.003 *SE*) μatom of oxygen were consumed in the absence of II, producing a P:O ratio of 1.94. In the presence of II, 0.41 (±0.04 *SE*) μmole of inorganic phosphate and 0.66 (±0.03 *SE*) μatom of oxygen were used, producing a P:O ratio of 0.61, *i.e.*, 31% of the control values.

Effect of II and 2,4-Dinitrophenol on Adenosine Triphosphatase Activity—The adenosine triphosphatase activity of mitochondria was determined by the literature method (23). Compound II was dissolved in 10 m*M* tromethamine hydrochloride buffer (100 μmoles/ml), and 2,4-dinitrophenol was dissolved in absolute ethanol (200 μmoles/ml). The media (1.5 ml), as described in the literature (23), were added to a number of centrifuge tubes (15 ml), and varying concentrations of II or 2,4-dinitrophenol were added to each tube. The reaction was commenced by the addition of a suspension of mitochondria in 3.4 m*M* tromethamine hydrochloride buffer (pH 7.4) (0.5 ml containing 2 mg protein/ml), and the combined reaction mixture was incubated at 37° for 15 min. After quenching with ice-cold trichloroacetic aid (2 ml, 10% w/v), the reaction mixture was centrifuged at 35,000 rpm (average radius of 8.5 cm) for 5 min to remove protein. The liberated inorganic phosphate in the supernate was determined using a modification (22) of a literature method (24) involving measurement[7] of the absorbance at 700 nm.

[5] Sigma type 3, Sigma Chemical Co., St. Louis, MO 63178.
[6] Pye Unicam SP1700 uv spectrophotometer.
[7] Model 250 spectrophotometer with a rapid sampler (model 2443-A), Gilford Instrument Laboratories Inc., Oberlin, OH 44074.

The amounts of inorganic phosphate liberated at varying concentrations of II, given in parentheses, were as follows. All figures indicate micromole quantities, and those prefaced by a negative sign indicate that the inorganic phosphate concentration was less than the control values, *i.e.*, inhibition of latent adenosine triphosphatase activity occurred: −0.0243 (0.20), 0.0845 (0.40), 0.1874 (0.60), 0.3256 (0.80), 0.3462 (1.00), 0.5086 (1.20), 0.3844 (1.40), 0.4535 (1.60), 0.4322 (1.80), 0.2412 (2.00), 0.0074 (2.50), −0.0784 (3.00), −0.1874 (4.00), and −0.2058 (5.00). The amounts of inorganic phosphate liberated when 2,4-dinitrophenol was used in place of II were as follows: 0.7754 (0.20), 0.5604 (1.00), 0.5898 (2.00), 0.5990 (3.00), −0.3675 (10.00), and −0.4116 (20.00). The figures obtained were the average values of six determinations.

Screening of Compounds—Details of the anticancer screening of II, IIIa, and IIId were described earlier (3). Compounds IIIb and IIIc were evaluated by the Drug Research and Development Division of the National Cancer Institute, Bethesda, Md., using their protocols (25), and were administered as a suspension in saline with polysorbate 80[8] by the intraperitoneal route on 9 successive days using male CD_2F_1 mice and male $B_6D_2F_1$ mice, respectively. The maximum increases in median survival times were 13% (100 mg/kg) for IIIb and 5% (100 mg/kg) for IIIc. There were five out of six survivors on Day 5 when IIIc was administered at 200 mg/kg and six out of six survivors on Day 5 at a dose of 100 mg/kg. No mortalities for IIIb were noted at a dose of 200 mg/kg.

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Pharmacokinetic Interactions of Tolazamide and Oxyphenbutazone in Dogs

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Abstract □ The described pharmacokinetic analysis involved two separate studies on nine dogs randomly assigned to three groups of three dogs each. In the first study, the effect of varying the dosage of tolazamide was examined. The second study concerned the effect of varying the dosage of oxyphenbutazone on tolazamide. A 3 × 3 Latin square was used to study both effects. Each group received each treatment, with a minimum of 1 week separating each session. The pharmacokinetics of tolazamide followed a two-compartment open model. The hybrid rate constants, α and β, were not significantly different at the three dosages when measured by a three-way analysis of variance. The only significant difference at the three dosage levels of tolazamide was in the apparent volume of distribution. In the pharmacokinetic interaction associated with intravenous administration of one dose of tolazamide and three doses of oxyphenbutazone, the apparent volume of distribution and the hybrid rate constant α did not change significantly while the hybrid rate constant for tolazamide, β, seemed to decrease with increasing oxyphenbutazone.

Keyphrases □ Pharmacokinetics—tolazamide and oxyphenbutazone interactions, dogs □ Tolazamide—pharmacokinetic interactions with oxyphenbutazone, dogs □ Oxyphenbutazone—pharmacokinetic interactions with tolazamide, dogs

Enhanced hypoglycemic effects have been reported when patients, stabilized on a sulfonylurea (tolbutamide or tolazamide), have had phenylbutazone and/or its analog, oxyphenbutazone, added to the treatment regimen (1). Hussar (2) suggested that phenylbutazone/oxyphenbutazone enhanced the hypoglycemic effects of tolbutamide or tolazamide.

Other investigators (3) found significant prolongation of the half-life of tolbutamide when phenylbutazone was administered at the same time. However, there have been few studies on this pharmacokinetic interaction and its clinical significance. None of the studies dealt with the pharmacokinetic parameters involved in the interaction of oxyphenbutazone and tolazamide when administered simultaneously.

There are three possible mechanisms for a pharmacokinetic interaction that enhances the hypoglycemic effects of tolazamide and other sulfonlyureas in the presence of phenylbutazone or oxyphenbutazone:

1. Inhibition of drug metabolism, *i.e.*, a decrease in metabolism of tolazamide/sulfonylurea, which could be caused by the presence of oxyphenbutazone or phenylbutazone.

2. Displacement of tolazamide/sulfonylurea from protein binding sites, which could result in increased blood concentrations of unbound, pharmacologically active tolazamide/sulfonylurea or its metabolites and could also produce high urinary concentrations of unchanged drug and its metabolites.

3. Inhibition of the excretion of tolazamide/sulfonylurea by a direct effect on the kidney.

The purpose of this study was to determine if a measurable pharmacokinetic interaction existed between tolazamide and oxyphenbutazone and which mechanism might explain it.

EXPERIMENTAL

Experimental Design—A 3 × 3 Latin square was used to study the effect of varying dosage on tolazamide pharmacokinetics. A similar Latin square design was used for the tolazamide–oxyphenbutazone interaction study, in which the dose of tolazamide was kept constant and the doses of oxyphenbutazone were varied.

Nine dogs were randomly assigned to three groups of three dogs each. Each group received each treatment, with a minimum of 1 week of rest separating each session.

The study was conducted over 3 months, with the sequence of treatments in accordance with a Latin square design (Table I). In the first study, Treatments A, B, and C were 5, 20, and 35 mg of tolazamide/kg, respectively. In the second study, Treatments A, B, and C were 10, 15, and 20 mg of oxyphenbutazone/kg, respectively, all with 20 mg of tolazamide/kg.

Typical Sampling Times—Zero time for tolazamide sampling was taken to be the midpoint of the tolazamide injection. Sampling times were 2, 5, 10, and 30 min and 1, 2, 4, 6, 8, 12, and 24 hr after the tolazamide injection.

Animals—Nine mongrel dogs, 9.0–16.5 kg, were obtained from the local animal shelter. These animals were vaccinated and given appropriate treatment for parasites; after acclimation, they were certified as healthy.

A catheter was surgically placed in the jugular vein so that the tip was

Table I—Latin Square Design for the Study of Tolazamide Pharmacokinetics

Group	Dog Identification Number	Week 1	Week 2	Week 3
1	1, 2, 3	A	B	C
2	4, 5, 6	B	C	A
3	7, 8, 9	C	A	B

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